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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Yasuo Koishihara

Title: INHIBITOR OF LYMPHOCYTE ACTIVATION

Appl. No.:   09/760,723

Filing Date: 01/17/2001

Examiner: G. Ewoldt

Art Unit: 1644

SUPPLEMENTAL RESPONSE UNDER 37 C.F.R. § 1.111

Mail Stop NON-FEE AMENDMENT
Commissioner for Patents
PO Box 1450
Alexandria, Virginia 22313-1450

Sir:

This is a supplemental response to the Non-Final Supplemental Office Action mailed on September 22, 2003, concerning the captioned patent application. A first response was filed on December 22, 2003.

Receipt of Priority Documents

The Supplemental Office Action indicated that the Office has not received certified copies of the priority documents.

Applicant believes, however, that the Office received these documents from the International Bureau as part of this application's parent case, U.S. App. No. 09/367,833. As evidence that the Office received the priority documents, Applicant has attached (a) a Notification of Acceptance of Application under 35 U.S.C. 371 and 37 C.F.R. 1.494 or 1.495, and (b) a Notice Informing the Applicant of the Communication of the International Application to the Designated Offices. The former indicates that by October 21, 1999, the Office had received a copy of the parent international application in English and a copy of the

Japanese priority application; the latter indicates that by September 3, 1998, the International Bureau had forwarded the parent international application to the Office.

Accordingly, Applicant respectfully requests that the Office reconsider whether the requirements for foreign priority have been satisfied.

Copy of Cited Document

In the first response, Applicant cited Ohtomo *et al.*, Biochem. Biophys. Res. Commun. 258(3): 583-591 (1999) and attached an abstract of that paper for the Examiner's consideration. Applicant now has obtained and submits for consideration a clean copy of the complete paper.

Concluding Remarks

Applicant believes that this application is in condition for allowance, and respectfully requests favorable reconsideration of it. If the Examiner believes that an interview would advance prosecution, he is invited to contact the undersigned by telephone.

The Commissioner is hereby authorized to charge any fees that may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date _____ *Jan. 8, 2004* _____   _____

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Molecular Cloning and Characterization of a Surface Antigen Preferentially Overexpressed on Multiple Myeloma Cells

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HM1.24 antigen has been identified as a surface molecule preferentially expressed on terminally differentiated B cells, and its overexpression is observed in multiple myeloma cells. The HM1.24 antigen is, therefore, expected as a most potent target molecule for antibody-based immunotherapy for multiple myeloma. Here, we have identified the cDNA for human HM1.24 antigen and also analyzed its gene structure including the promoter region. The HM1.24 antigen is a type II membrane glycoprotein, which has been reported as a bone marrow stromal cell surface antigen BST2, and may exist as a homodimer on myeloma cell surface. Although a reason for the overexpression in myeloma cells is not understood, very interestingly, the promoter region of the HM1.24 gene has a tandem repeat of three cis elements for a transcription factor, STAT3, which mediates interleukin-6 (IL-6) response gene expression. Since IL-6 is a differentiation factor for B cells, and known as a paracrine/autocrine growth factor for multiple myeloma cells, the expression of HM1.24 antigen may be regulated by the activation of STAT3. Importantly, a humanized anti-HM1.24 antibody effectively lysed the CHO transformants which expressed HM1.24 antigen as high as human multiple myeloma cells, but not the cells with lower antigen expression. This evaluation shows that ADCC heavily depends on the expression level of target antigens and, therefore, the immunotherapy targeting the HM1.24 antigen should have a promising potential in clinical use. © 1999 Academic Press

Multiple myeloma (MM) is a lethal disease characterized by a clonal accumulation of plasma cells and usually accompanied by homogeneous immunoglobulin in the serum and/or urine. Bone marrow invasion by these tumor cells is associated with severe anemia and humoral immunodeficiency resulting in concomitant bacterial infections (1). In addition, an abnormal cytokine environment such as elevated IL-6 and/or IL-1β as well as TNFα, often results in increased osteolysis leading to bone pain, pathologic fractures, and hypercalcemia (2). Since a variety of chemotherapies have had little impact on the overall clinical course, alternative approaches against multiple myeloma are greatly needed (3). Indeed, several mouse monoclonal antibodies have been established to target cell surface antigens such as CD38 (4, 5) and CD54 (6) on myeloma cells, or been shown to inhibit the factor-dependent cell growth of MM cells (7). Furthermore, humanized anti-CD38 (8), anti-interleukin-6 (IL-6) and anti-IL-6 receptor antibodies (9–11) have shown their potential in therapeutic use against MM.

However, CD38 and CD54 are also found on a variety of cells including hematopoietic stem cells (12, 13), leading to serious reservations that these molecules can not be suitable target antigens for immunotherapy of plasma cell dyscrasias in terms of adverse effects. Furthermore, although IL-6 has been reported to be a major growth factor of MM cells, some myeloma cells do not proliferate in response to IL-6, and the inability of anti-IL-6 antibody to block proliferation of certain MM cells is known (14, 15).

To develop a novel immunotherapeutic strategy, we have generated a mouse monoclonal antibody, which is highly specific for a surface antigen of multiple my-

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eloma cells by immunizing a human plasma cell line KPC-32 (16). Flow cytometric and immunohistochemical analyses with this antibody showed that the antigen, designated HM1.24, is preferentially expressed on mature B cells, but is not or only slightly on other cells such as bone marrow, liver, spleen, kidney, heart, and so on. Surprisingly, the HM1.24 antigen is overexpressed in all multiple myeloma cells examined, suggesting this antigen as a good target for immunotherapy. Indeed, the mouse anti-HM1.24 antibody and its humanized derivative exhibit potent antitumor cell activity against multiple myeloma cells by inducing antibody-dependent cell-mediated cytotoxicity (ADCC) both in vitro and in vivo (17, 18).

In order to characterize the HM1.24 antigen, first, we identified the cDNA for human HM1.24 antigen from myeloma cells and analyzed its structural features, and then isolated the mouse and rhesus counterparts and carefully compared their structures. Next, we have analyzed the genomic DNA sequences of these HM1.24 antigens, and identified cis-elements which may be important for regulation of the HM1.24 gene expression. Furthermore, we have evaluated its potent as a target antigen for immunotherapy by establishing CHO cell lines which express HM1.24 antigen at different levels.

MATERIALS AND METHODS

Myeloma cell lines. Human multiple myeloma cell lines, RPMI8226 (ATCC CCL-155) and U266 (ATCC TIB-196) were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS) (Life Technologies, Rockville, MD), and KPMM2 (19) in the presence of IL-6 (2ng/ml) in RPMI 1640 with 20% FCS.

Immunoprecipitation and Western blot analysis. Cells were solubilized by sonication in a lysis buffer (50 mM sodium borate, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40) containing a protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany). After centrifugation, soluble fractions were collected and incubated with Sepharose 4B beads (Pharmacia Biotech, Uppsala, Sweden) conjugated with either mouse anti-HM1.24 (16) or anti-BST2 (20) antibodies. The beads were precipitated by a brief centrifugation and boiled for 5 min in Laemmli's sample buffer to release the bound proteins. The proteins were separated on gradient (4–20%) SDS-polyacrylamide gels, and then transferred onto PVDF membranes (Millipore, Bedford, MA). Following pretreatment for nonspecific binding, the membranes were incubated with the anti-HM1.24 or anti-BST2 antibodies. The membranes were incubated with POD-labeled anti-mouse IgG and visualized by the ECL system (Amersham, Buckinghamshire, UK).

Construction of cDNA libraries. Total RNA was isolated from KPMM2 cells by a standard guanidium thiocyanate/cesium chloride method, and mRNA was purified by a FirstTrack mRNA purification kit (Invitrogen, Carlsbad, CA). First strand cDNA was synthesized with a NotI/Oligo-dT$_{18}$ primer, and an EcoRI adapter was added to the double stranded cDNA (the TimeSaver cDNA Synthesis Kit; Pharmacia Biotech). The cDNA larger than 0.7kb was size-fractionated by electrophoresis on a 1% low melting agarose gel. Following digestion with NotI, the cDNA was ligated into pCHO-1 (21) derivative, pCOS-1 expression vector for panning with COS-7

cells (Library A), and a λExCell vector (Pharmacia Biotech) for immunoscreening with E.coli (Library B).

Panning. Panning plates were prepared as follows. Each 3 ml of the antibody solution (10 μg/ml of mouse anti-HM1.24 antibody in 50 mM Tris-HCl buffer, pH 9.5) was added to four 6 cm tissue culture dishes and incubated for 2 h at room temperature. Following washes with 0.15 M NaCl, 3 ml of PBS containing 5% FCS, 1 mM EDTA and 0.02% NaN$_3$ was added to the dishes, and incubated for 2 h at room temperature.

Library A was used in a panning screen according to a standard protocol (22). In brief, 20 μg of plasmid DNA containing 5 × 10^5 independent cDNA clones were transfected into COS-7 cells by an electroporation method. The transfected cells were divided into four 10 cm tissue culture dishes and cultured for 72 h in DMEM supplemented with 10% FCS, and then washed with PBS and harvested with PBS containing 5 mM EDTA. The cells were suspended in PBS containing 5% FCS and 0.02% NaN$_3$ to a concentration of 1 × 10^6 to 2 × 10^6 cells/ml.

The cells were divided into the four panning dishes. After 2 h incubation at room temperature, the plates were gently washed three times with 3 ml of PBS containing 5% FCS and 0.02% NaN$_3$. The cells remaining on the dishes were harvested and plasmid DNAs were recovered from the cells by a Hirt method (23).

Immunoscreening. Library B (approximately 2.2 × 10^5 clones) was subjected to an immunoscreening using a *pico*Blue Immunoscreening kit (Stratagene, La Jolla, CA) according to a standard protocol (24).

Human genomic DNA cloning. PCR primers were designed based on the cDNA (P3.19) encoding a full-length HM1.24 antigen. Human genomic DNA segments coding for HM1.24 antigen were PCR-amplified from a human peripheral leukocyte genomic DNA (Clontech # 6550-1). The promoter region was also PCR-amplified by a PromoterFinder DNA walking kit (Clontech, Palo Alto, CA). The PCR products were subcloned into a pCRII cloning vector (Invitrogen), and DNA sequences were determined.

Mouse HM1.24 cloning. The human HM1.24 cDNA was subjected to a BLAST homology search. Two mouse ESTs, AA097629 and AA117071, were identified and then a full length cDNA was PCR-amplified from mouse myeloma MOPC21 (ATCC TIB-9) cells based on the DNA sequences of the ESTs.

Rhesus HM1.24 cloning. A Rhesus liver genomic library (Clontech #OL1004d) was screened by a plaque hybridaization with a ^{32}P-labeled human HM1.24 cDNA fragment under stringent conditions. Positive clones which hybridized with both the 3′ and 5′ regions of the human cDNA were selected by a Southern blot analysis. The DNA sequence of a selected clone was determined, and carefully compared with those of human cDNA and genomic DNA. Based on the similarity of the human and rhesus DNA sequences, four exons were easily identified to yield a putative rhesus HM1.24 cDNA sequence.

Construction of recombinant CHO cell lines. In order to establish CHO transformants which stably express human HM1.24 antigen, the cDNA (P3.19) was transfected into CHO cells, and transformants were selected in the presence of 500 μg/ml of G418 (Life Technologies). In the same manner, an empty vector, pCOS1, was also introduced into CHO cells, and control CHO transformants were obtained.

Flow cytometric analysis. CHO cells were stained with either anti-HM1.24 or anti-BST2 mouse monoclonal antibodies and FITC-labeled goat anti-mouse Ig, and then analyzed on a FACScan (Becton Dickinson & Co., Mountain View, CA) according to a standard protocol (16).

Preparation of a soluble HM1.24 antigen. A soluble HM1.24 antigen (Asn-49 to Gln-180) was designed to remove the putative cyto-
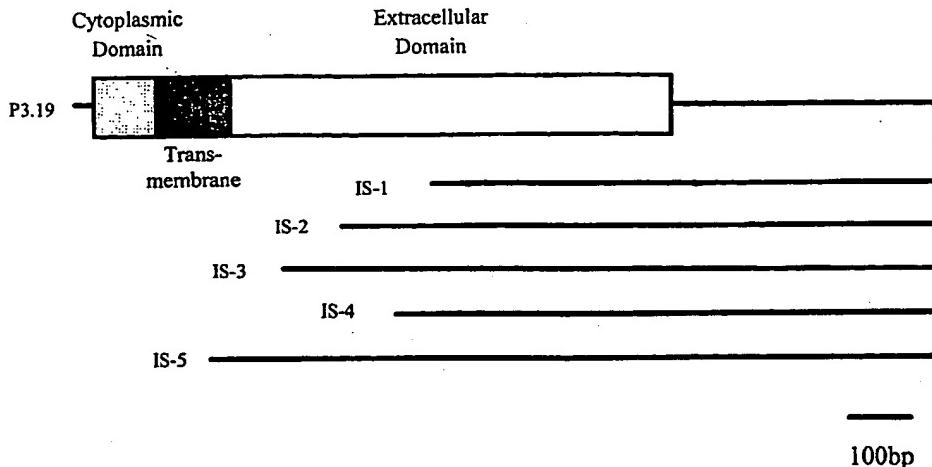
FIG. 1. Schematic representation of cDNA structure for human HM1.24. A full-length cDNA clone P3.19 (1014 bp) encoding the HM1.24 antigen was isolated by a panning screen with COS-7 cells. IS-1 to IS-5 were obtained from an immunoscreening.

plasmic and transmenbrane domains, and an HA-tag was added to its N-terminus. A leader peptide from immunoglobulin was used for its efficient secretion into culture medium. The DNA sequence encoding this fusion protein was inserted into a pCHO1 expression vector.

The culture supernatants from CHO transformants with the expression vector were subjected to a SDS-polyacrylamide gel electrophoresis. Proteins on the gel were transfered onto a PVDF membrane. The soluble HM1.24 antigen was visualized by the ECL Western blot detection system in the same manner as mentioned above.

ADCC assay. Peripheral blood mononuclear cells (PBMCs) were prepared from a healthy volunteer by density gradient centrifugation with Ficoll-Paque Plus (Pharmacia Biotech). After washing with RPMI1640 (Life Technologies) containing 10% FCS, PBMCs were used as effector cells according to a standard 4 h ^{51}Cr-release assay, as described below. A human myeloma cell lines, KPMM-2 (19) and three transformed CHO cell lines, were incubated in the presence of Na$_2$ ^{51}CrO$_4$ (100 μCi/10^6 cells) for 1h at 37°C, and then washed with RPMI1640/10% FCS. The reaction (200 μl) in the 96-well plate contained the effector cells and the ^{51}Cr-labeled target cells at varying E/T ratios, 50/1 to 0/1, and various amounts of antibodies. After incubating for 4 h at 37°C, supernatants were harvested and their radioactivity read on a gamma counter. Spontaneous release of ^{51}Cr was determined by incubating the target cells in the absence of the effector cells, and maximum ^{51}Cr-release was obtained by lysing the cells in 1% NP40. The specific lysis (%) was calculated from the formula: 100 × (experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release).

RESULTS

Human HM1.24 Antigen

An approximately 1 kb cDNA, designated P3.19 (Figure 1), was specifically enriched after four rounds of panning from the initial of 5 × 10^5 independent clones. To confirm the binding of anti-HM1.24 antibody to a protein encoded by P3.19, CHO cells were transformed with P3.19, and analyzed by a flow cytometry. The anti-HM1.24 antibody strongly bound to the transformed cells with P3.19,

but not with a control vector pCOS1 (Figure 2a and 2b), indicating that P3.19 encodes the HM1.24 antigen. In addition, all five positive cDNA clones (IS-1 to IS-5) isolated by immunoscreening corresponded to the DNA sequence of P3.19 (Figure 1).

A database search with the cDNA sequence revealed that P3.19 is a known cDNA encoding BST2, which was isolated as a bone marrow stromal cell surface molecule (20). To confirm this result, three multiple myeloma cell lines were subjected to immunoprecipitation-western blot analyses in which anti-HM1.24 and anti-BST2 antibodies were combined. The anti-BST2 antibody recognized immunoprecipitates by the anti-HM1.24 antibody, and vice versa (Figure 3A and 3B). Similar results were obtained from the CHO transformants as described above. In addition, the anti-BST2 antibody bound to the CHO transformant with P3.19 as well as the anti-HM1.24 antibody in a flow cytometric analysis (Figure 2c and 2d).

Based on the deduced amino acid sequence, the HM1.24 antigen is characterized as a type II membrane protein with a Mr of approximately 24 kDa. There are two potential acceptor sites for N-linked carbohydrates and three cysteine residues in the extracellular domain (Figure 4). The recombinant HM1.24 antigen expressed on the CHO cells showed a heterogeneity with a Mr of 29-33 kDa under reduced conditions, similar to the antigen naturally expressed on myeloma cells (Figure 3A), supporting the idea that the HM1.24 antigen is heterogeneously glycosylated. Indeed, several lectins with different specificities against oligosaccharide bound to the HM1.24 antigen (data not shown). In addition, under the unreduced conditions, the immunoprecipitates from the CHO transformants showed a higher Mr of 50 to 66 kDa (Figure 3A), suggesting that the
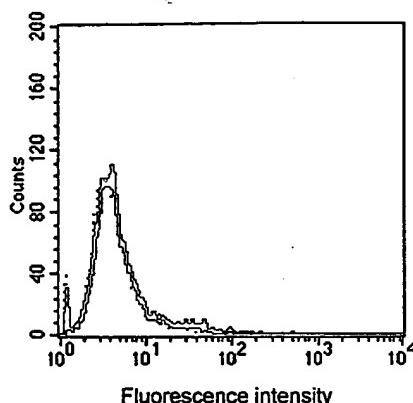
(a)   CHO/NEO (Anti-HM1.24)   (b)   CHO/HM (Anti-HM1.24)
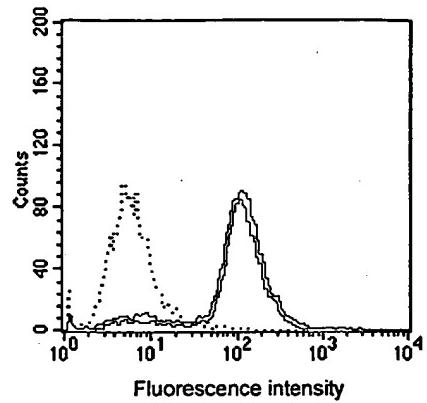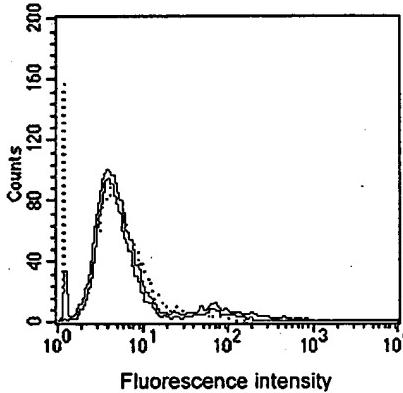
(c)   CHO/NEO (Anti-BST2)   (d)   CHO/HM (Anti-BST2)
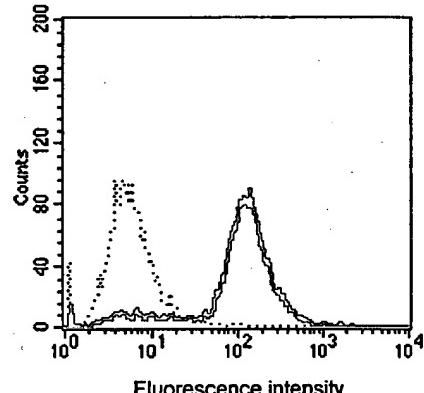
FIG. 2.   Flow cytometric analyses of transformed CHO cells. CHO/HM (b, d) and CHO/NEO (a, c) are transformed cells with P3.19 and a pCOS1 empty vector, respectively. These cells were incubated with either mouse anti-HM1.24 antibody (16) (a, b) or mouse anti-BST2 antibody (20) (c, d), and added FITC-labeled goat anti-mouse Ig (solid lines). An isotype matched control mouse antibodies were used in each analysis (dotted lines).

HM1.24 antigen may exist as a homodimer rather than a heterodimer via disulfide bond in the extracellular domain. As evidence to support this idea, a soluble HM1.24 antigen can be purified as a homodimer from the culture supernatant of transformed CHO cells with a mutant construct in which the cytoplasmic and transmembrane regions were removed (Figure 3C).

Mouse and Rhesus HM1.24

A BLAST homology search identified two mouse ESTs, AA097629 (394 bp) and AA117071 (322 bp), which showed 57.4% and 55.9% identities, respectively, with the cDNA of human HM1.24 antigen. Based on the DNA sequences of the ESTs, a full length cDNA was successfully PCR-amplified from mouse myeloma MOPC21 cells. The cDNA and its

deduced amino acid sequences show 51.3% and 39.8% identities, respectively, with those of the human HM1.24 antigen.

Four exons were easily identified in the rhesus genomic sequence by comparing with those in the human HM1.24 gene, and then assembled to generate a putative rhesus HM1.24 cDNA. The cDNA and the deduced amino acid sequences show 86.0% and 77.2% identities, respectively, with those of the human HM1.24 antigen.

Figure 4 shows a sequence alignment of human, rhesus, and mouse HM1.24 antigens. Three cysteine residues and two N-glycosylation sites in the extracellular domain are completely conserved even in mouse, and interestingly, a cytoplasmic consensus sequence, Tyr-(X)-Tyr-(X)$_3$-Pro-Met, was found in their short intracellular domains.

(A)
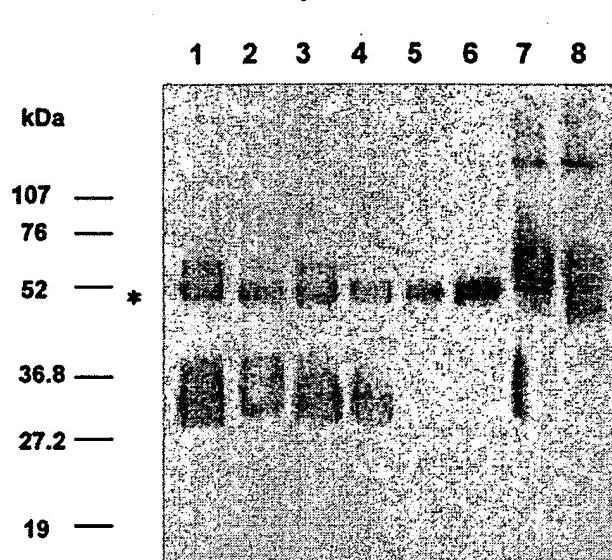
(B)
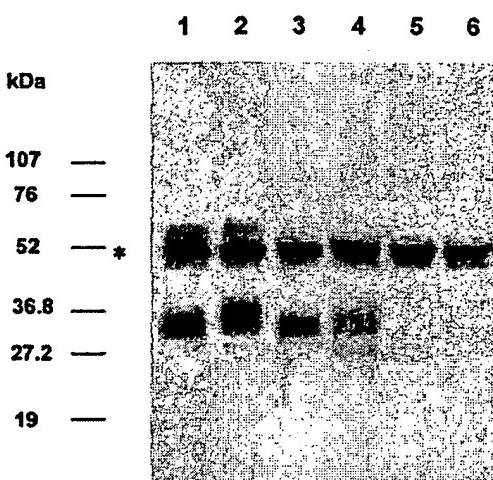
(C)
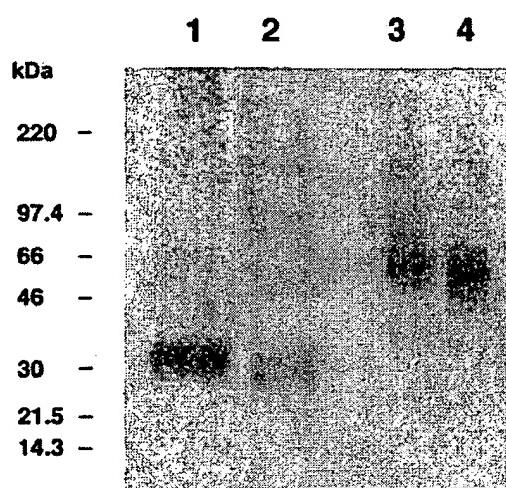
FIG. 3. Western blot analyses for human HM1.24 antigen. (A) KPMM2 (1.0×10^6 cells; lane 1, 7), U266 (5.0×10^6 cells; lane 2), RPMI8226 (5.0×10^6 cells; lane 3), CHO/HM transfected with P3.19 (5.0×10^6 cells; lane 4, 8), CHO/NEO, control transfectant (5.0×10^6 cells; lane 5), and none (lane 6) were subjcted to an immunoprecipition with mouse anti-HM1.24 antibody. Following SDS-PAGE under the reduced (lane 1-6) or the unreduced conditions (lane 7, 8), a blotted membrane was stained with mouse anti-BST2 antibody. (B) The same cells used in (A) (lanes 1-6) were immunoprecipitated with mouse anti-BST2 antibody, and the same manner, a blotted membrane was stained with mouse anti-HM1.24 antibody. An asterisk * indicates a nonspecific band derived from the immobilized immunogloblin heavy chain. Molecular weight standards in kilodaltons are shown to the left. (C) Culture supernatant of the CHO cells which express a soluble construct of HM1.24 antigen (lane 2, 4) were analyzed with cell lysate prepared from KPMM2 (lane 1, 3) as positive controls under the reduced (lane 1, 2) or the unreduced conditions (lane 3, 4).

ADCC Analysis

In order to evaluate its potent as a target antigen for immunotherapy, several CHO cell lines which express HM1.24 antigen at different levels were established and compared in the ADCC assay (Figure 5). A FACS analysis showed that CHO/30 cells expressed the HM1.24 antigen at almost equal level of human myeloma KPMM2 cells, in which the binding site of anti-HM1.24 antibody was estimated to be approximately 10^5 sites per cell (16). The antigen expression of CHO/1 and CHO/21 cells were roughly estimated as 1/10 and 1/200 of the CHO/30 cells.
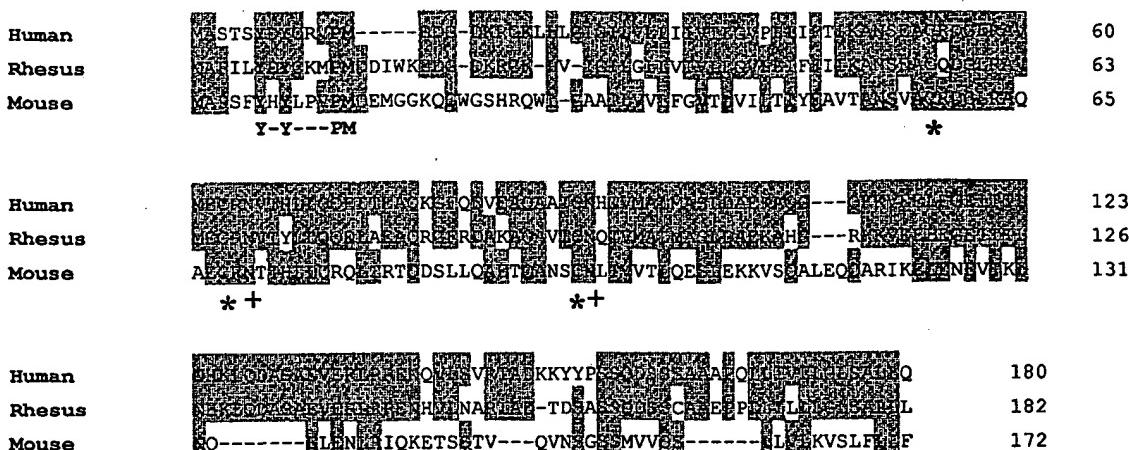
FIG. 4. A multiple alignment of human, rhesus and mouse HM1.24 antigens. Identical amino acid residues in two/three spices are boxed by dark. Putative acceptor sites of N-linked carbohydrates, N-X-T/S, are indicated with +, and cysteine residues conserved in the extracellular domain are marked by an asterisk. A cytoplasmic consensus sequence, Tyr-(X)-Tyr-(X)$_3$-Pro-Met, are shown in bold letters.

CHO/30 and CHO/1 (roughly estimated 10^4–10^5 molecules/cell) were efficiently lysed by even at 0.1 μg/ml of the humanized anti-HM1.24 antibody. This sensitivity for ADCC was very similar to those of human myeloma cells such as KPMM2 and ARH77 (17, 18). Whereas CHO/21 (low level) did not show any sensitivity even at 10 μg/ml of the antibody. These data supports the fact that ADCC activities heavily depends on the density/expression level of target antigens.

Analysis of the HM1.24 Promoter Region

The genomic structure of HM1.24 antigen consists of four exons in approximately 2.5 kb. In order to analyze why HM1.24 antigens are over-expressed on myeloma cells, its promoter region of approximately 2 kb was PCR-amplified and sequence-analyzed. First, a putative transcription initiation site was determined as follows. HM1.24 cDNAs were synthesized by a Cap-Finder PCR cDNA construction kit (Clontech), which is reported to efficiently synthesize full-length cDNAs by the CapSwitch oligonucleotide. Based on the sequence of eight HM1.24 cDNA clones, the transcription may initiate from the A at 51 bp upstream from the ATG translation initiation codon. Indeed, a TATA box like sequence (TAATAAA) was found at approximately 30bp upstream from the putative transcription initiation site (Figure 6).

Next, to find cis elements for transcription factors, the human promoter region was computer-analyzed with the TFSEARCH. As shown in Figure 6, a tandem repeat of three IL-6 response type II element/APRF site (25, 26), CTGG(G/A)AA, was found at positions −146 to −135 from the transcription initiation site. This element is also known as the STAT3 binding site (27, 28). The tandem repeat also contains IFN response elements IRF-1/2 (29), AAAAG(T/C)GAAA, and ISGF3

(30), AGTTTCNNTTTCN(C/T), and GAS (31), TTNC-NNNAA. Moreover, several IL-6 response type I elements/NF-IL6 sites (32), T(T/G)NNGNAA(T/G), was also found, although they are relatively distant from the transcription initiation site and dispersed over the 2 kb promoter region (data not shown). Another feature of interest in the promoter region is that there are several AP-2 binding sites (33).

DISCUSSION

In this study, we have isolated the cDNA for the HM1.24 antigen to understand its properties as a myeloma specific surface antigen. The cDNA cloning revealed that the HM1.24 antigen is identical to a known type II membrane protein which was previously isolated as a bone marrow stromal cell surface antigen, BST2 (20). A mutant construct, in which the N-terminal cytoplasmic and transmembrane regions were removed, was efficiently secreted as a homodimer molecule into a culture supernatant of the CHO transformant, suggesting that the HM1.24 antigen may be expressed as a homodimer molecule on myeloma cell surface.

A sequence alignment of mouse, rhesus, and human HM1.24 antigens well conserve the characteristics of the cysteine residues and N-glycosylation sites in the extracellular regions, and interestingly, a consensus sequence, Tyr-(X)-Tyr-(X)$_3$-Pro-Met, in the short cytoplasmic regions. Although biological functions of HM1.24 are not yet understood, this cytoplasmic domain would likely play a role in transducing intracellular signaling. Therefore, experimental approaches focusing on the consensus sequence such as a tyrosine phosphorylation study or a two-hybrid screen to analyze associated molecules will be important into characterizing the biological functions of HM1.24.

(A)
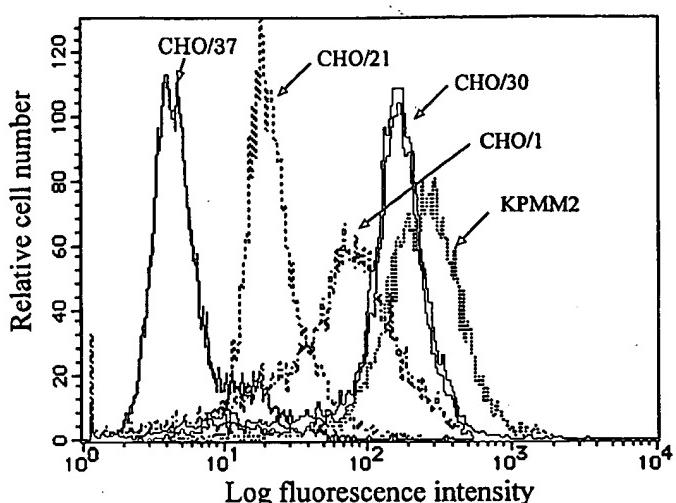
(B)
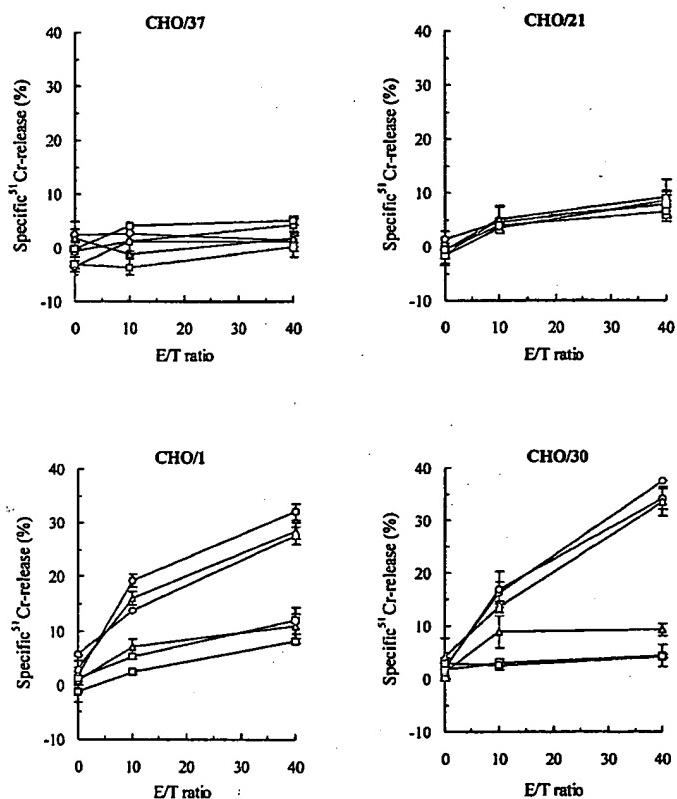
FIG. 5. ADCC activity of humanized HM1.24 antibody against CHO transformants. (A) Several clonal cell lines from CHO transformants were established, and selected in terms of their expression level of HM1.24 antigen by a FACS analysis. Three cell lines, CHO/21 (low level), CHO/1 (medium level), and CHO/30 (high level) were from the transformants with P3.19, and CHO/37 (antigen −) from those with the empty vector. As a positive control, human KPMM2 myeloma cells which express HM1.24 antigen at the level of approximately 10⁵ molecules per cell was analyzed. (B) ⁵¹Cr-labeled CHO transformed cells were incubated with human peripheral blood mononuclear cells at different E/T ratio in the presence of 10 μg/ml

On the other hand, it was reported that fibroblast cell lines transformed with BST2 facilitated the stromal cell-dependent cell growth of mouse bone marrow-derived pre-B DW34 cells, which suggests that BST2 may be involved in pre-B cell growth via cell-cell interaction. This also suggest that there is a receptor or ligand for BST2/HM1.24 antigen. The transgenic mouse (overexpression and gene-targeted) is currently under construction, which will help elucidate the biological functions of the HM1.24 antigen.

IL-6 is a differentiation factor in the B cell development (34, 35), and has been shown to be a paracrine/autocrine growth factor for multiple myeloma (15, 36, 37). It has been also reported that IL-6 triggers phosphorylation of STAT1 and/or STAT3 in multiple myeloma cells independent of their proliferative response to IL-6 (38). In the promoter region of the HM1.24 gene, a tandem repeat of three cis elements for STAT3, which also contains IFN response elements, IRF-1/2, ISGF3, and GAS, was found approximately 150bp upstream from the transcription initiation site. Activation of STATs, specially STAT3 by IL-6, seems likely to be mainly involved in the regulation of HM1.24 expression on B cells, and the constitutive activation of STATs may cause the overexpression of the HM1.24 antigen in multiple myeloma cells.

Additionally, several AP-2 sites are identificible in the promoter region. AP-2 has been reported to be activated by TPA, cAMP and retinoic acid (37), and to be able to activate p21$^{WAF1/CIP1}$ expression, consistent with a potential role for AP-2 in differentiation (39). It would be expected that AP-2 is involved in B cell differentiation, and regulates HM1.24 expression in cooperation with STAT3. However, an alignment of the DNA sequences of human, mouse and rhesus promoter regions (approximately 350 bp) revealed that the tandem repeat of STAT3 rather than the AP-2 sites is well conserved in these promoters (data not shown). This would support the idea that STATs are major transcription factors regulating the gene expression of the HM1.24 antigen. A detailed analysis of the HM1.24 promoter region will identify indispensable cis-elements and transcription factors.

Very importantly, the humanized anti-HM1.24 antibody effectively lysed the CHO transformants which expressed HM1.24 antigens as high as human multiple myeloma cells. As previously reported, the anti-HM1.24 antibody bound terminally differentiated B cells, but its reactivity was very low compared with myeloma cells. Furthermore, memory B cells, early stage B cells, and another types of cells were

(O), 1 μg/ml (O), 0.1 μg/ml (△) and 0.01 μg/ml (△) of the humanized (RVLa/RVHr) antibody (20). Human IgG1 10 μg/ml (□) was used as a negative control. Antibody (−) was shown by (□).

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-500 AGAGGCACTGGATGAAGCCCTGCTCGTCACCACAGAGACACCTGAACACAAAAACCAGTCCCTGGGGTCA
                               CREB
-430 GACCCAGGCCCCGCCCCCAGACCCAGGCCCTGCCCTCACTCCACCACGCAACTGTGCAACCTCAGTTTCC
         AP-2  Sp1,AP-2        AP-2
-360 CCAGGTGGAGACCGGACCAACAATGATGGCCTCTGCCTCTTCAGGTCATAGTACAGATGAATACAGGCTG
-290 GCACGGCCTAGGCACTCAGTAACACACGGCAGAGGCACAGGGACTTAAGATGGAGTGTCCCAGGCAGCCA
-220 CAGTTGGCTGGCACCCAGTTGGGAAGGGCCCAAGGGCTTTTAAAGCAGGGTGAAAAAAAAAGCCCACCTC
         STAT3      STAT3     STAT3        AP-2
-150 CTTT▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨▨CCTAATTAATCCTCTGCCTGTAGGTGCCTCATGCAAGAGCTGCTG
            ISGF3,IRF1/2
 -80 GTCAGAGCACTTC▨▨▨▨▨▨▨TTGCTATTGGTCAGGACGTTTCCTATGCTAATAAAGGGGTGGCCCGTAGA
              STAT3                                   AP-2
               ┌─→
 -10 AGATTCCAGCACCCTCCCCTAACTCCAGGCCAGACTCCTTTCAGCTAAAGGGGAGATCTGGATGGCATCT
                                                             M  A  S
 +60 ACTTCGTATGAC
      T  S  Y  D
```

FIG. 6. Nucleotide sequence of the 5′ flanking promoter region of human HM1.24 gene. A putative transcription initiation start site (+1) is indicated by an arrow, and a TATA like sequence, TAATAAA, at −32 to −26 are boxed. The cis elements for transcription factors, AP-2, CREB, SP1 are underlined, and for STAT3, ISGF3, and IRF-1/2 are boxed and shaded. A translation initiation ATG codon is at position +51.

little or not reacted with the antibody in the FACS analyses. Therefore, the immunotherapy inducing ADCC against the HM1.24 antigen should have a promising potential in clinical use for multiple myeloma patients.

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